

High Glucose Up-Regulates ENaC and SGK1 Expression in HCD-Cells

¹Claire E. Hills, ¹Rosemary Bland, ¹Jeanette Bennett, ²Pierre M. Ronco and ¹Paul E. Squires

¹Molecular Physiology, Biomedical Research Institute, Department of Biological Sciences, University of Warwick, Coventry, ²Unité INSERM 702; Université Paris 6, Hôpital Tenon (AP-HP), Paris

Key Words

SGK1 • ENaC • TGF β • Sodium • Hyperglycaemia • Kidney

Abstract

Background/Aim: Diabetic nephropathy is associated with progressive renal damage, leading to impaired function and end-stage renal failure. Secondary hypertension stems from a deranged ability of cells within the kidney to resolve and appropriately regulate sodium resorption in response to hyperglycaemia. However, the mechanisms by which glucose alters sodium re-uptake have not been fully characterised. **Methods:** Here we present RT-PCR, western blot and immunocytochemistry data confirming mRNA and protein expression of the serum and glucocorticoid inducible kinase (SGK1) and the α conducting subunit of the epithelial sodium channel (ENaC) in a model *in vitro* system of the human cortical collecting duct (HCD). We examined changes in expression of these elements in response to glucose challenge, designed to mimic hyperglycaemia associated with type 2 diabetes mellitus. Changes in Na⁺ concentration were assessed using single-cell microfluorimetry. **Results:** Incubation with glucose, the Ca²⁺-ionophore ionomycin

and the cytokine TGF- β 1 were all found to evoke significant and time-dependent increases in both SGK1 and α ENaC protein expression. These molecular changes were correlated to an increase in Na⁺-uptake at the single-cell level. **Conclusion:** Together these data offer a potential explanation for glucose-evoked Na⁺-resorption and a potential contributory role of SGK1 and ENaCs in development of secondary hypertension, commonly linked to diabetic nephropathy.

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Introduction

Regulation of renal sodium (Na⁺) handling is a key determinant in the long-term control of extracellular fluid volume homeostasis. Na⁺-resorption is mediated via the amiloride-sensitive epithelial sodium channel (ENaC) that exhibits high selectivity for sodium [1] and is a central requirement for Na⁺-resorption across renal epithelia. ENaC expression and translocation to the plasma-membrane is tightly regulated by a diverse array of hormonal [2-5] and physical factors [6, 7]. Key in the regulation of ENaC function is the serum and glucocorticoid-regulated kinase (SGK1). SGK1 is known

to phosphorylate the neural precursor cell expressed developmentally down-regulated ubiquitin protein ligase Nedd 4.2 [8, 9]. Normally bound to the ENaC in its unphosphorylated form, SGK1-mediated phosphorylation causes Nedd 4.2 to release ENaC to the membrane consequently allowing for increased Na⁺-uptake. These mechanisms partially explain immediate increases in transepithelial sodium uptake in response to acute, but physiologically appropriate stimuli. Longer-term regulation is likely to be dependent on the de novo synthesis of both SGK1 and ENaC.

In type 2 diabetes mellitus, insulin insensitivity results in fluctuating levels of hyperglycaemia, and in extreme cases; glucosuria. Previous reports have already established the deranged transcriptional regulation of SGK1 in response to hyperglycaemia [10]. These elevated circulating levels of glucose have also been associated with the release of a number of growth factors and cytokines known to be involved in promoting the pathogenesis of diabetic nephropathy, amongst these is the Transforming Growth Factor-β1 (TGF-β1) [11-14]. Hyperglycaemic-induced TGF-β1 formation together with osmotically-driven increases in SGK1 [15-17] provide a link between poorly controlled plasma glucose and the development of excess ENaC-mediated Na⁺-resorption that underlies secondary hypertension as seen in some diabetics (reviewed in Marshall et al, 2004). In addition to these glucose mediated effects on TGF-β1 driven by numerous factors including PKC [18] and the cell volume regulatory responses to cell shrinkage initiated as a result of hyper-osmotic stress, glucose is also known to acutely increase the concentration of intracellular calcium ([Ca²⁺]_i) in rat proximal tubular cells [19]. How these calcium induced changes evoke alterations in both SGK1 and ENaC expression in human cortical collecting duct (HCD) remains to be elucidated. Consequently, whilst there is considerable evidence relating high glucose levels to deranged sodium resorption in the kidney, the mechanisms by which these elements come together to promote the pathophysiological changes characteristic of diabetic nephropathy requires further clarification.

In the current study we have used a novel model in vitro system to assess the effect of high glucose, TGF-β1 and cytosolic Ca²⁺ on SGK1 and ENaC expression. We have correlated these changes to single-cell determination of [Na⁺]_i and suggested a potential series of events that may explain why deregulated Na⁺ re-uptake may have dramatic repercussions for normal kidney function, contributing to the pathogenesis of secondary hypertension associated with diabetic

nephropathy in type 2 diabetes mellitus.

Materials and Methods

Cell Culture

HCD cells were derived from normal human kidney cortex and immortalized with SV-40 virus. Clones were selected using the monoclonal antibody, Ab272, which specifically recognizes collecting duct principal cells [20]. HCD cells (passages 18-30) were maintained in DMEM/Hams F-12 medium (GIBCO, Invitrogen), supplemented with 2% fetal calf serum (FCS), glutamine (2mmol/l), 15mmol/l HEPES, transferrin (5μg/ml), Na₂SeO₃ (5ng/mL), insulin (5μg/ml) and dexamethasone (5x10⁻⁸ M). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air. For glucose experiments, cells were treated with 5mM or 25mM glucose in the presence of unsupplemented DMEM/Hams F-12 without FCS for time periods of 0, 24 and 48 hours following FCS-deprivation overnight. Basal (5mM) glucose culture media was generated by mixing DMEM (0mM glucose) with HAMS F-12 (10mM glucose). For TGF-β1 and Ionomycin experiments, cells were treated with either TGF-β1 (2nM) or ionomycin (1μM) in the presence of DMEM/Hams F-12 without FCS for periods of 4, 6, 8, 12 and 24 hours. For sodium experiments, cells were seeded onto 3-Aminopropyltriethoxy-silane (APES) (Sigma, Poole, UK) treated coverslips and used within 1 day of plating.

Analysis of mRNA expression

RNA was prepared from 80% confluent HCD cells by acid-guanidinium extraction [21] using a Genelute mammalian total RNA miniprep kit (Sigma) following the manufactures instructions. Complementary DNA was synthesized by reverse transcription using a Promega Reverse Transcription System following an adapted method. Briefly, 1μg of total RNA and 0.5μg of random hexamers, in a final volume of 11μl, were incubated at 70°C for 5 minutes, and then allowed to cool slowly to 25°C. Primer extension was then performed at 37°C for 60 minutes following the addition of 1x (final concentration) reaction buffer, containing 50mmol/l Tris-HCl (pH 8.3), 50mmol/l KCl, 10mmol/L MgCl₂, 10mmol/l dithiothreitol and 0.5mmol/l spermidine, 1mmol/l (final concentration) of each dNTP, 40U of rRNasin ribonuclease inhibitor and 15U of AMV reverse transcriptase in a final volume of 20μl. The RT mixture was heated to 95°C for 5 minutes, then 4°C for 5 minutes. An aliquot of 4μl was used in subsequent polymerase chain reaction (PCR) reactions.

PCR amplification of cDNAs

Amplification of specific cDNAs was carried out using the primers listed in Table 1. PCR reactions (20μl) were set up containing 1.5mmol/l MgCl₂, 0.2mmol/l of each dNTP, 0.5μM of each primer and 1U of Taq DNA polymerase (Bioline). Amplification of samples was performed using an initial denaturation step of 95°C (5 minutes) followed by either 35 cycles (SGK1) (TGF-β1), 30 cycles (αENaC) consisting of one minute of denaturing at 95°C, one minute of annealing at the

mRNA	Primers (5'→3')	Direction	Annealing temperature	Product size (bp)
SGK1	AGGGCAGTTTTGGAAAGGTT GCAGAAGGACAGGACAAAGC	F R	51	699
αENaC	CCAGCTACCAGCTCTCTGCT TTCTCACACCAAGGCAGATG	F R	50	601
TGF-β1	GGGACTATCCACCTGCAAGA CACGTGCTGCTCCACTTTTA	F R	58	341

Table 1. Polymerase chain reaction (PCR) primers used to amplify SGK1 and αENaC mRNA. F = forward primer, R = reverse primer.

required temperature and a one minute extension at 72°C. A final elongation step of 72°C for seven minutes was included in all PCR amplifications.

Analysis of protein expression

The preparation of cytosolic proteins and their subsequent separation by gel electrophoresis and electroblotting onto Immobilon P membrane (Millipore, Watford, UK) were as described previously [22]. Briefly proteins (5μg) were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (4.5% stacking gel, 7.5% or 10% resolving gel) at 200volts for 50 minutes in electrophoresis buffer containing 25mmol/l Tris, 192mmol/l glycine and 0.1% (wt/vol) SDS. Proteins were transferred onto Immobilon P membrane in transfer buffer (25mmol/l Tris, 192mmol/l glycine, 20% (vol/vol) methanol) for 1 hour at 100 volts; 4°C. Following protein transfer, membranes were blocked in PBS-T (PBS plus 0.1% Tween-20) containing 20% (wt/vol) nonfat milk powder (Marvel, Premier Brands, Stafford, UK) for 1 hour at 25°C and then washed with PBS-T for 15 minutes. Filters were analyzed with specific polyclonal antibodies against human SGK1 (The Binding Site) anti-human αENaC (Affinity Bioreagents) and anti-TGF-β1 (Santa Cruz) at a dilution of 1:3000 + 0.01% milk, 1:600 and 1:500 respectively. After three 10 minute washes in PBS-T, the membranes were incubated with the secondary antibody (horseradish peroxidase- conjugated) anti-rabbit (αENaC diluted 1:2000, TGF-β1 diluted 1:40,000) and anti-sheep (SGK1 diluted 1:30000) in PBS-T (0.05%) for 60 minutes at 25°C followed by three 10 minutes washes in PBS-T. Specific proteins were detected using ECL detection reagent chemiluminescence system (Amersham Biosciences) and were visualized after exposure of membranes to X-ray film for 1-10 minutes. Control experiments were included where primary antibody was omitted, and filters were exposed to secondary antibody and ECL detection. All blots were probed with GAPDH (Biogenesis, UK) diluted at 1:20,000 to control for protein loading in subsequent densitometry analysis of expression levels.

Immunocytochemistry

Cells were allowed to grow to 80% confluence on APES treated coverslips and then fixed in 4% paraformaldehyde (PFA). Non-specific binding was prevented by blocking for 1 hour at 25°C in PBS + 0.01% triton X-100 containing 10% normal

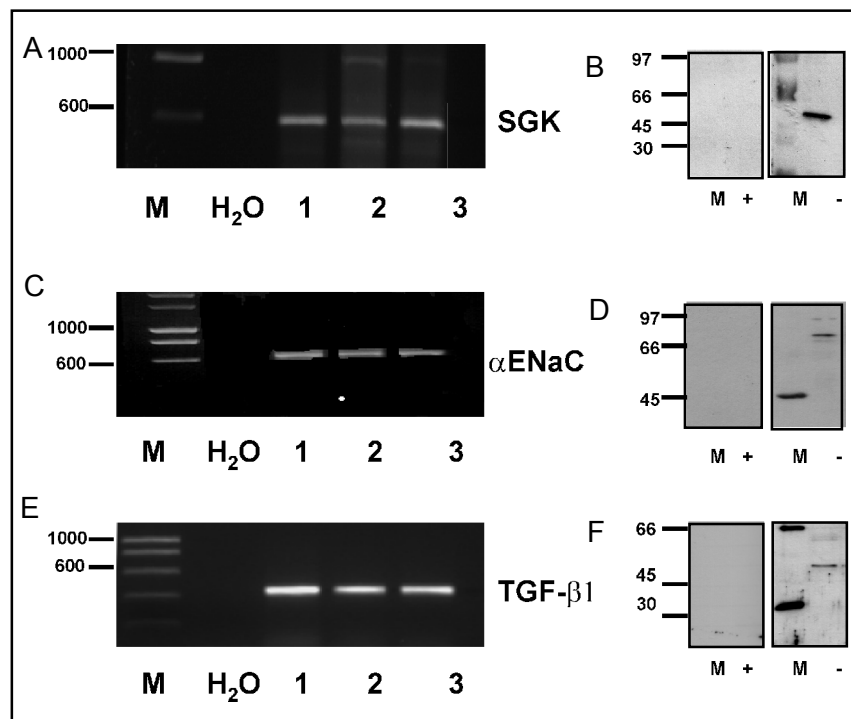
goat serum. Following three 10 minute washes with PBS, the nuclear stain DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; 1mmol/L) was added to each coverslip for 3 minutes. After washing with PBS (3x5 minutes), cells were incubated with the cytoskeletal stain TRITC-conjugated-Phalloidin (Sigma) diluted at 1:100 in PBS supplemented with 0.01% Triton X-100 for 1 hour at 25°C. Following further washing (3x5 minutes), cells were incubated overnight at 4°C with the corresponding primary antibody (anti-αENaC, anti-SGK1) diluted in PBS + 0.01% Triton X-100. Following antibody incubation, cells were washed with PBS and then incubated in Alexa 488 conjugated goat anti-rabbit (αENaC) or goat anti-sheep (SGK1) for 1 hour at 25°C. Secondary antibodies (Molecular Probes) were diluted (1:400) in PBS + 0.01% Triton X-100. After 1 hour, cells were washed 3x10 minutes and coverslips mounted in citifluor (glycerol/PBS solution: Agar Scientific) on glass slides.

Single cell Microfluorimetry

HCD cells seeded and grown overnight on APES-coated coverslips were loaded with 20 μl of the Na⁺-fluorophore SBF/Pluronic acid (20%) (Sigma). Cells were incubated for 90 minutes in a humidified atmosphere (5% CO₂) at (37°C). HCDs are a polarized, epithelial cell model, which compensate Na⁺ influx via apical epithelial sodium channels with Na⁺ efflux at the basolateral pole via a Na⁺/K⁺ ATPase. Therefore in order to resolve small changes in cytosolic Na⁺ concentration it was necessary to debilitate extrusion mechanisms in both control and experimental conditions. Cells were incubated with the Na⁺/K⁺ ATPase pump inhibitor Ouabain (100μM) for the last 30 minutes of the incubation period. To determine whether the acute application of glucose had an effect on [Ca²⁺]_i cells were loaded with 2.5μM of the Ca²⁺-fluorophore fura-2/AM (30 minutes, 37°C; Sigma).

Coverslips were washed and placed in a steel chamber, the volume of which was approximately 500μl. A single 22mm coverslip formed the base of the chamber, which was mounted into a heating platform on the stage of an Axiovert 200 Research Inverted microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). All experiments were carried out at 37°C using unsupplemented DMEM/Hams F-12 as the standard extracellular medium. Cells were illuminated alternatively at 340nm and 380nm using a Metaflour imaging workbench (Universal Imaging Corp Ltd.,

Fig. 1. Expression of SGK1, α ENaC and TGF- β 1 mRNA and protein in HCD-cells. Panels A, C and E represent RT-PCR analyses using primers specific for human SGK1, α ENaC and TGF- β 1. PCR products of 597bp, 618bp and 340 bp were observed in three RNA preparations (1, 2 and 3) corresponding to mRNA expression for SGK1, α ENaC and TGF- β 1 respectively in HCD cells. Negative controls included both water and samples in which AMV enzyme had been omitted from the reverse transcription reaction (data not shown). Western blot analyses of HCD cell lysates (panels B, D and F, 5 μ g protein/lane) using an antibody against human SGK1 (panel B, -), α ENaC (panel D, -) and TGF- β 1 (Panel F, -) confirmed the presence of the protein in HCD cells. A protein band of approximately 50kDa (SGK1), 75Da (α ENaC) and 45 kDa (TGF- β 1) was detected. Controls included SGK1 (panel B +), α ENaC (panel D +) and TGF- β 1 (Panel F +) antibody pre-absorbed with a 10-fold excess of immunizing peptide.



Marlow, Bucks, UK). Emitted light was filtered using a 510nm long-pass barrier filter and detected using a Cool Snap HQ CCD camera (Roper Scientific). Changes in the emission intensity of SBF and fura-2 expressed as a ratio of dual excitation were used as an indicator of changes in $[Na^+]_i$ using established procedures. Data was collected at 3 second intervals for multiple regions of interest in any one field of view. All records have been corrected for background fluorescence (determined from cell-free coverslip).

Data Analysis

Autoradiographs were quantified by densitometry (TotalLab 2003). Statistical analysis of data was performed using a one-way ANOVA test with a Tukey's Multiple Comparison post-test. Data are expressed as arithmetic mean \pm SEM and n denotes the number of experiments and $P < 0.05$ signifies statistical significance.

Results

Expression of SGK1, α ENaC and TGF- β 1 in HCD cells

Studies confirmed the presence of SGK1, α ENaC and TGF- β 1 mRNA and protein in HCD cells. RT-PCR analysis of several RNA preparations from HCD cells revealed PCR products representative of SGK1, α ENaC and TGF- β 1 mRNA (figure 1A, 1C and 1E respectively). To confirm that mRNA was appropriately translated,

protein expression was determined by Western blotting (figures 1B, 1D and 1F). Western blot analyses revealed bands at approximately 50kDa, 75kDa and 45kDa, representative of those expected for SGK1, α ENaC and TGF- β 1 respectively.

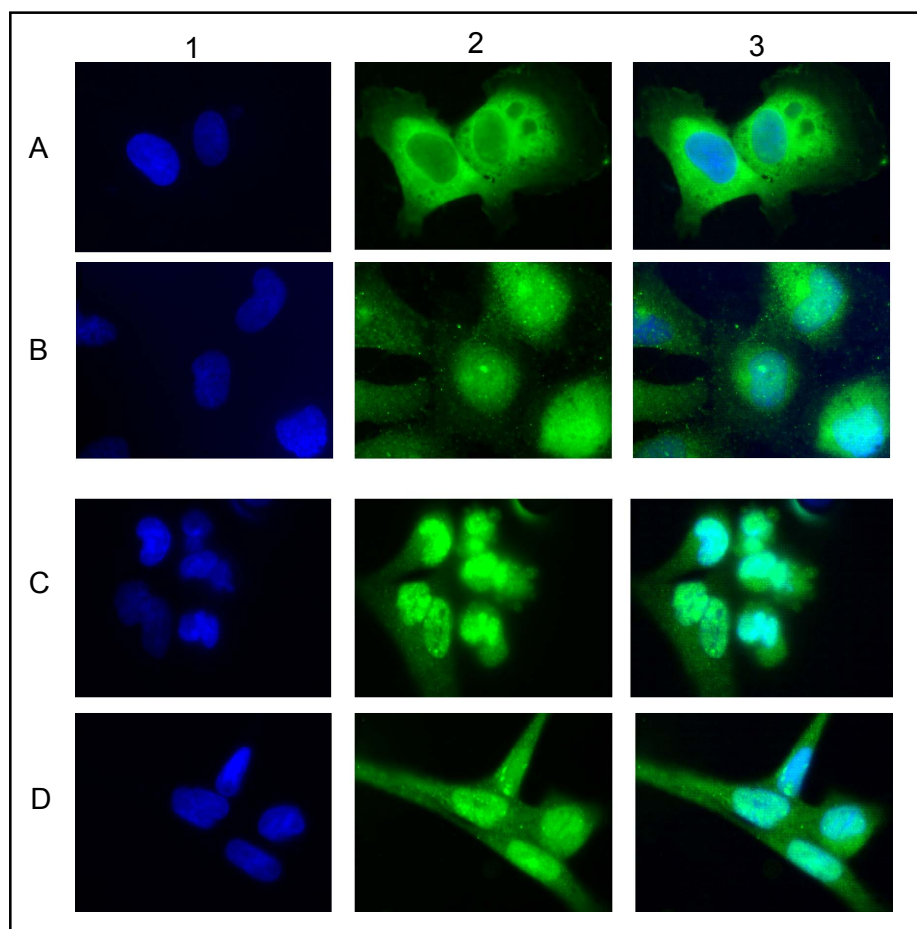
The effect of glucose on the localisation of SGK1 and α ENaC

The distribution of SGK1 and α ENaC in HCD cells was examined by immunocytochemistry. SGK1 protein was principally localised to the cytosol (figure 2 A/2), whilst α ENaC immunoreactivity appeared to be predominantly nuclear (figure 2 C/2). Following 48 hour incubation with high glucose (25mM) SGK1 redistributed into the nucleus (figure 2 B/2) whilst α ENaC was found in both the nucleus and the cytoplasm under identical conditions (figure 2 D/2).

Up-regulation of SGK1 and α ENaC expression in high glucose

To examine the effect of elevated glucose on SGK1 and α ENaC expression, HCD cells were incubated in high glucose (25mM) for 24 and 48 hours. Cells grown under these conditions exhibited increased SGK1 expression to $185 \pm 18.8\%$ of control (5mM) at 24 hours and to $261.8 \pm 5.7\%$ of control at 48 hours ($n=3$, $P < 0.01$; see figure 3 A and B). HCD cells grown under high

Fig. 2. Altered localization of SGK1 and α ENaC in response to high glucose. SGK1 immunoreactivity in HCD-cells following a 48hr culture is shown in row A (5mM glucose) and B (25mM glucose), whilst α ENaC immunoreactivity is shown in row C (5mM glucose) and D (25mM glucose) over the same 48hr time interval. Column 1 demonstrates nuclear staining (DAPI; blue); column 2 illustrates either SGK1 (A and B) or α ENaC (C and D) immunoreactivity (localized using Alexa 488; green). Column 3 depicts an overlay image. Note the intense cytosolic localization of SGK1 at sub-stimulatory glucose concentrations. SGK1 re-distributes to the nucleus in response to elevated (25mM) glucose. The localization of α ENaC is primarily nuclear under resting conditions, and becomes more cytosolic in response to high glucose.



glucose conditions also exhibited increased α ENaC expression at both 24 and 48 hours. This increase in protein expression amounted to $144 \pm 8.0\%$ of control (5mM) at 24 hours and $167.4 \pm 11.2\%$ of control at 48 hours ($n=3$, $P<0.01$; see figure 3 C and D).

Up-regulation of TGF- β 1 expression in high glucose

To examine the effect of elevated glucose on TGF- β 1 expression, HCD cells were incubated in high glucose (25mM) for 24 and 48 hours. Cells grown under these conditions exhibited increased TGF- β 1 expression to $130 \pm 16.7\%$ of control (5mM) at 24 hours and to $187.7 \pm 15.8\%$ of control at 48 hours ($n=3$, $P<0.01$; see figure 4 A and B).

Up-regulation of SGK1 expression in response to elevated calcium levels and TGF- β 1

Previous reports have suggested that raised $[Ca^{2+}]_i$ and increased circulating levels of the cytokine TGF- β 1 play a role in the patho-physiology of hyperglycaemia. In the current study 25mM glucose was found to evoke a rise in TGF- β 1 expression at 48 hours, $187.7 \pm 8.4\%$ of

control ($n=3$, $P<0.01$; data not shown). The glucose-evoked up-regulation of SGK1 has been linked to these signaling intermediates [23]. Treatment of HCD cells with the Ca^{2+} ionophore ionomycin ($1\mu M$ see figure 5A and B) significantly increased SGK1 expression to $262 \pm 6.0\%$ of control at 6 hours and $263 \pm 22.8\%$ of control at 8 hours ($n=3$, $P<0.01$), with expression levels returning to near basal by 24 hours. However the effect of TGF- β 1 (figure 5C and D) was less potent with expression only increased at 8 hours $128 \pm 13\%$ as compared to control ($n=4$, $P<0.01$). Co-application of TGF- β 1 and ionomycin failed to augment the change in expression, (data not shown).

Up-regulation of alphaENaC expression in response to elevated calcium levels and TGF- β 1

Treatment of HCD cells with ionomycin ($1\mu M$) resulted in increased α ENaC expression levels to $175 \pm 5.5\%$ of control at 6 hours (figure 6A and B, $n=3$; $P<0.01$). Whilst expression was clearly elevated above basal levels at both 4 ($135 \pm 1.0\%$) and 8 hours ($136 \pm 7.3\%$) expression was not found to be statistically significant. The administration of TGF- β 1 also elicited a

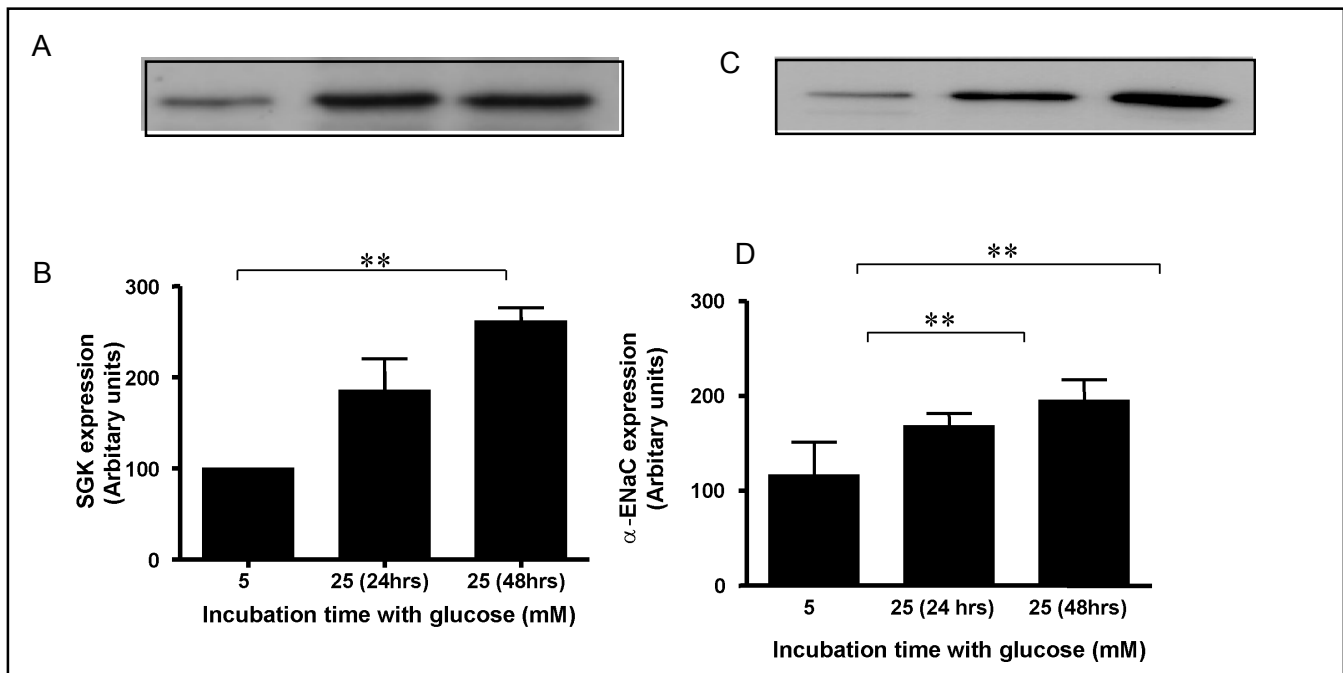


Fig. 3. Up-regulation of SGK1 and α ENaC and protein expression in response to high glucose. HCD-cells were incubated in 5mM and 25mM glucose for 24 and 48 hours. (A) Representative Western blot analysis using an anti-SGK1 antibody (B) analysis of changes in SGK1 protein expression. Results represent mean \pm SEM; $n=4$; $** P < 0.01$. (C) Representative Western blot analysis using an anti- α ENaC antibody (D) analysis of changes in α ENaC protein expression. Results represent mean \pm SEM; $n=4$; $** P < 0.01$.

stimulatory effect over α ENaC expression at 6 hours, $159.7 \pm 9.8\%$ (figure 6C and D, $n=3$; $P < 0.01$) as compared to control. Co-application of TGF- β 1 and ionomycin failed to augment the change in expression, (data not shown). Together, these data suggest a role for both $[Ca^{2+}]_i$ and TGF- β 1 in the regulation of SGK1 and α ENaC protein expression.

HCD-cells exposed to high glucose exhibit increased $[Na^+]_i$ and $[Ca^{2+}]_i$

Acute (5 minute) application of glucose (20mM) evoked a small and sustained increase in $[Ca^{2+}]_i$ (27 cells in 4 separate experiments). The effect was identical to that produced by a comparable concentration (20mM) of mannitol (33 cells in 4 separate experiments), used to control for the osmotic effect of high glucose.

Intracellular sodium levels were found to be significantly elevated following 24 ($113.1 \pm 1.0\%$) and 48 ($114 \pm 1.1\%$) hours exposure to 25mM glucose, as compared to cells cultured in low (5mM) glucose ($n=3$ $P < 0.01$, see figure 7). This data taken in conjunction with that described previously suggest that increases in $[Na^+]_i$ expression in response to high glucose after 48 hours may be mediated via the increased expression of both SGK1 and α ENaC.

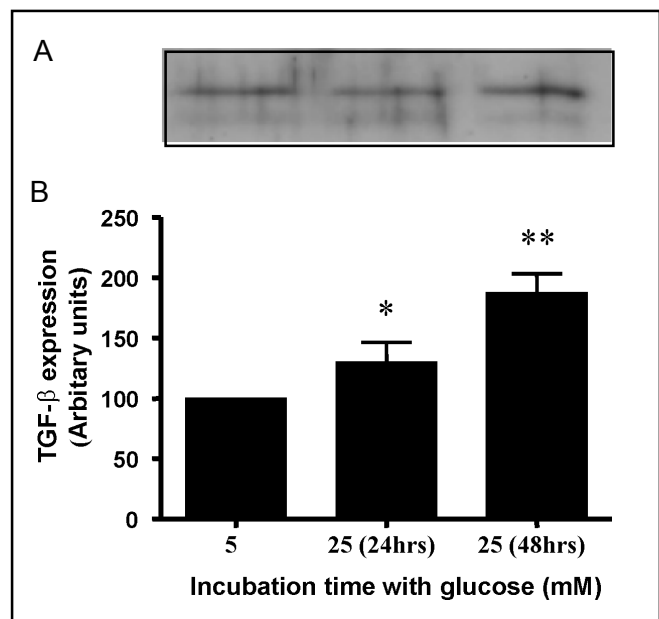


Fig. 4. Up-regulation of TGF- β 1 protein expression in response to high glucose. HCD-cells were incubated in 5mM and 25mM glucose for 24 and 48 hours. (A) Representative Western blot analysis using an anti-TGF- β antibody (B) analysis of changes in TGF- β 1 protein expression. Results represent mean \pm SEM; $n=6$; $* P < 0.05$, $** P < 0.01$.

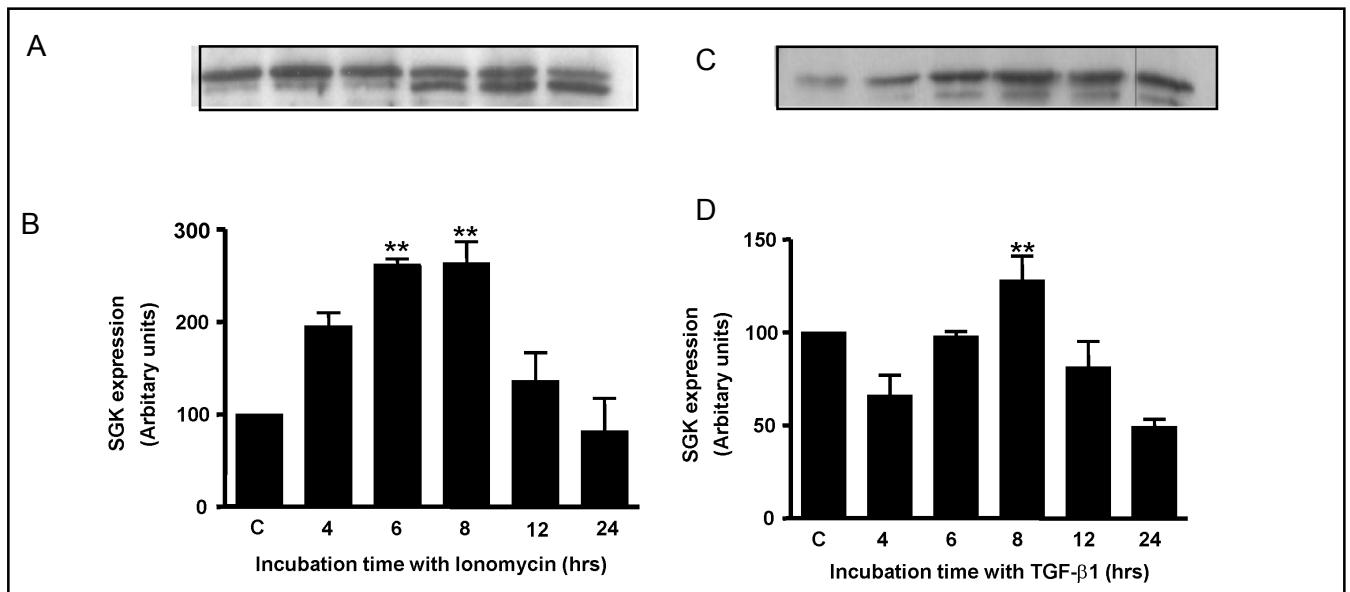


Fig. 5. Treatment of HCD cells with ionomycin and TGF- β 1 results in increased SGK1 protein expression. HCD cells were incubated with either ionomycin (1 μ M) or TGF- β 1 (2 nM) for incubation periods of 4, 6, 8 12 and 24 hours. (A and C) Representative Western blot analysis using an anti-SGK1 antibody (B and D) analysis of changes in SGK1 protein expression. Results represent mean \pm SEM; n=4; ** P < 0.01.

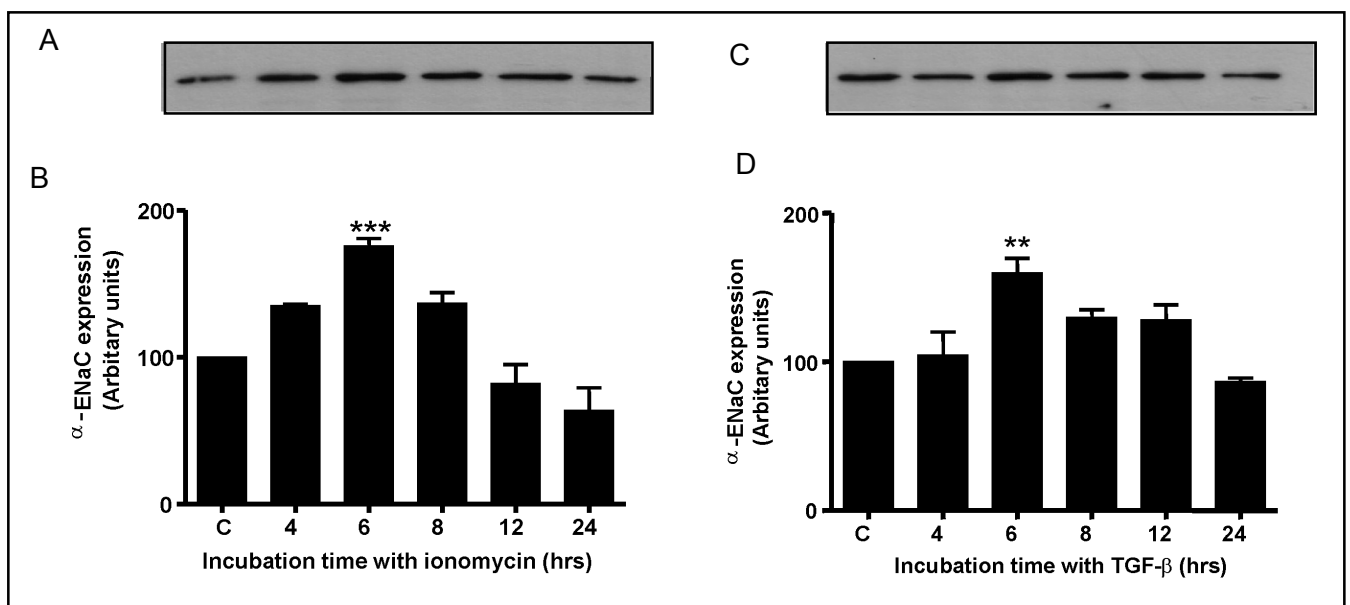


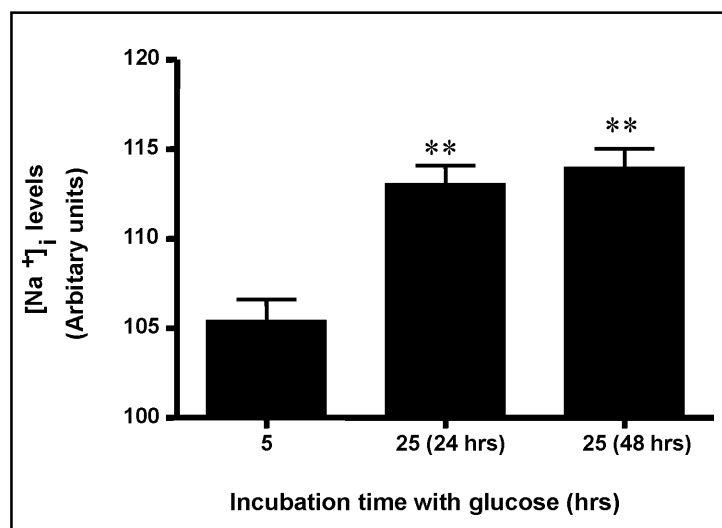
Fig. 6. Treatment of HCD cells with ionomycin and TGF- β 1 results in increased α ENaC protein expression. HCD cells were incubated with either ionomycin (1 μ M) or TGF- β 1 (2 nM) for incubation periods of 4, 6, 8 12 and 24 hours. (A and C) Representative Western blot analysis using an anti- α ENaC antibody (B and D) analysis of changes in α ENaC protein expression. Results represent mean \pm SEM; n=4; ** P < 0.01, *** P < 0.001.

Discussion

Exposure of renal epithelial cells to changes in osmolality necessitates the initiation of cell volume regulatory processes, activation of which serves to restore cell volume preserving both integrity and function. Inability

to resolve these changes may have serious repercussions for sodium transport and the development of secondary hypertension associated with renal diseases such as diabetic nephropathy. In the current study we have used a model *in vitro* system of the human cortical collecting duct (HCD) to investigate expression of key recognition

Fig. 7. HCD cells exposed to elevated levels of glucose exhibit increased intracellular sodium levels. HCD cells were incubated in 5 or 25mM glucose for 24 and 48 hours. Cells were then pre-incubated with the Na⁺/K⁺ ATPase inhibitor Ouabain. Basal levels of intracellular Na⁺ were measured in SBF-1/pluronic loaded HCD cells and variability in [Na⁺]_i between glucose treatment was assessed. Data represent mean±SEM, n=3; ** *P* < 0.01.



signaling elements involved in Na⁺ reabsorption under control and high glucose conditions.

We present data confirming mRNA and protein expression of the serum and glucocorticoid inducible kinase (SGK1) and the α conducting subunit of the epithelial sodium channel (ENaC) in HCD-cells. Incubation with glucose, the Ca²⁺-ionophore ionomycin and the cytokine TGF- β 1 were all found to evoke a time-dependent increase in both SGK1 and α ENaC protein expression. These molecular changes, seen under conditions of high glucose, were correlated to an increase in Na⁺-uptake at the single-cell level. High glucose also alters the cellular localization of both SGK1 and α ENaC. Together these data offer a potential explanation for glucose-evoked Na⁺-resorption and the contributory role of SGK1 and ENaCs in development of secondary hypertension, commonly linked to diabetic nephropathy.

Transfer of information regarding the osmotic state of the duct depends largely on cell-to-cell coupling. Consequently, changes in intercellular communication may play a key role in transducing changes in osmolarity to appropriate changes in absorption.

We have recently reported that HCD-cells express the mechano-sensitive transient receptor-potential channels, TRPV4 [24]. These channels respond to osmotic perturbations by allowing Ca²⁺-entry and store-mobilization, a pre-requisite for initiation of a regulatory volume decrease [25, 26], instigated following exposure to hypo-osmotic stress to aid cell volume recovery [27]. Resultant Ca²⁺-signals are rapidly synchronized across coupled cells via connexin-43-mediated gap-junctions [24]. However, cytosolic Ca²⁺ does not merely synchronize cellular activity between HCD-cells. Hyperglycaemia has been associated with elevated

cytosolic calcium in a number of diverse cell types from both animal and human models of diabetes [19, 28, 29]. In the present study, acute application of glucose (20mM) evoked a small, but sustained increase in cytosolic calcium in HCD-cells, whilst direct elevation of [Ca²⁺]_i via ionomycin treatment significantly increased SGK1 protein expression after 6-8hrs, a finding consistent with previous studies in DAN-G pancreatic tumor cells [30]. Similarly, glucose-mediated SGK1 transcription in 3T3 mouse fibroblasts is reportedly abolished in the presence of the calcium channel blocker nifedipine [10]. The metabolism of glucose can be ascribed to a number of pathways which are all interlinked [31]. In addition to evoking increases in [Ca²⁺]_i, glucose also increases the *de novo* synthesis of diacylglycerol (DAG) eliciting downstream activation of protein kinase C/mitogen activated protein kinase (PKC/MAPK) [32]. PKC has emerged as a potential regulator in the development and progression of diabetic nephropathy and reportedly plays a key role in TGF- β expression through its actions on the transcription factor complex AP-1 [33]. In accordance with previous literature, elevated [Ca²⁺]_i and signalling molecules arising from glycolytic intermediates, may regulate transcriptional control of SGK1 as downstream components of the hyperglycaemic response.

In the current study high glucose elevates TGF- β 1 expression at 24 and 48 hours. TGF- β 1 is a multifunctional cytokine known to be increased in type 2 diabetics and is thought to be involved in the development of renal hypertrophy and excessive matrix deposition, implicating this cytokine as a key mediator in the development of diabetic nephropathy [34-36]. TGF- β 1 reportedly alters SGK1 gene transcription [10], an effect believed to be mediated via p38 MAPK [15, 17]. This may further

exacerbate glucose-evoked cell hypertrophy, highlighting a possible link between elevated levels of circulating glucose and increased expression of SGK1. This relationship is supported in the current study by our observed changes in SGK1 expression following exogenous application of the cytokine, an effect known to be reduced in the presence of a TGF- β neutralizing antibody [10]. The temporal relationship between Ca^{2+} and TGF- β -evoked increases in SGK1-expression are consistent with previous reports highlighting a causative link between these key signaling elements [10]. It is well established that glucose increases the expression of TGF- β 1 [12, 13, 37], and TGF- β 1 increases expression of the insulin-independent GLUT 1 glucose transporter [38]. Consequently, GLUT1-mediated increases in TGF- β 1 expression will promote and further exacerbate the rate of glucose uptake under conditions of hyperglycaemia, contributing to the pathogenesis of diabetic nephropathy. This feed forward build up of TGF- β 1 may provide the link between gluco-toxicity and cell dysfunction in diabetic nephropathy, with TGF- β 1 mediating glucose-evoked cellular hypertrophy whilst stimulating matrix biosynthesis [39], two hallmarks of diabetic renal disease.

Whilst the transient nature of the TGF- β 1-evoked response may reflect glucose toxicity associated with exogenous application of high concentrations of the cytokine, perforation of the cell membrane by the Ca^{2+} -ionophore is likely to have multiple non-specific effects that ultimately impair cell function. These effects may explain why SGK1-expression appears to respond to changes in Ca^{2+} and TGF- β 1 before elevated glucose, even though both are downstream of hyperglycaemia.

SGK1 promotes Na^+ re-uptake via the epithelial sodium-channel (ENaC). This heterotrimeric channel resides in the cytoplasm; bound to Nedd 4.2, [40], and is

comprised of an α , β and γ subunit, of which only the α -subunit is required for formation of a fully functional channel. Phosphorylation of SGK1 via a PI3-K dependent pathway facilitates binding of Nedd 4.2 to SGK1, thereby dissociating the Nedd 4.2/ENaC complex. This dissociation reduces ENaC degradation and facilitates translocation and insertion of the channel into the cell membrane [8]. The association between SGK1 and ENaC leads us to assume that these glucose-, Ca^{2+} - and TGF- β 1-evoked changes in α ENaC should mimic those already reported for SGK1, an assumption supported by the current study. The net effect of altered ENaC-expression would be an increase in Na^+ -resorption. It seems reasonable therefore to assume that any glucose-evoked increase in α ENaC expression may result from reduced degradation, as opposed to increased gene transcription. Although at the single-cell level these changes in Na^+ re-uptake are small, across the epithelium, they offer a potential mechanism to explain how high glucose may lead to the development of secondary hypertension commonly seen in diabetic nephropathy [41].

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